FOSETYL-AL IS EFFECTIVE AGAINST MUTANTS OF *PHYTOPHTHORA CAPSICI* RESISTANT TO METALAXYL

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ABSTRACT – Mutants of *Phytophthora capsici* resistant to the systemic fungicide metalaxyl were readily obtained by treating thalli with ultraviolet radiation. Frequency of recovery of mutants was high (nearly 20%). All the mutants except one were able to grow *in vitro* in the presence of 100 µg/ml metalaxyl. Two types of mutants were distinguished with pathogenicity either similar to that of the wild-type or greatly reduced. The metalaxyl resistant mutants tested exhibited a sensitivity to fosetyl-Al similar to that of the wild-type both *in vitro* and *in vivo*.

RÉSUMÉ – L'irradiation aux ultra-violets de thalles de *Phytophthora capsici* a permis l'obtention aisée de mutants résistants au métalaxyl à une fréquence élevée (environ 20%). Tous les mutants obtenus sauf un, sont capables de pousser *in vitro* en présence de 100µg/ml de métalaxyl. Deux catégories de mutants sont distinguées : l'une dont le pouvoir pathogène est semblable à celui de la souche sauvage et l'autre dont le pouvoir pathogène est fortement diminué. Les différents mutants testés présentent une sensibilité au phosétyl-Al semblable à celle de la souche sauvage *in vitro* et *in vivo*.

MOTS CLÉS : *Phytophthora capsici*, mutant, resistance, metalaxyl, fosetyl-Al, pathogenicity.

INTRODUCTION

The systemic fungicides most commonly employed in the control of Oomycetes are metalaxyl (Ridomil, N-(2-6 dimethyl phenyl)-N(methoxyacetyl)-alanine methyl ester) and fosetyl-Al (Aliette, aluminium tris-O-ethyl phosphonate). Development of field resistance to these fungicides may interfere with disease control and be of economic importance. The occurrence of metalaxyl-resistant field isolates has already been reported among *Phytophthora infestans* (DAVIDSE & al., 1981a-b; DOWLEY & O'SULLIVAN, 1981; COHEN & REUVENI,

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1983), *Plasmopara viticola* (LEROUX & CLERJEAU, 1985), and *Pseudoperonospora cubensis* (REUVENI & al., 1980; GEORGOPOULOS & GRIGORIU, 1981; KATAN & BASHI, 1981). Recently, a cross resistance to metalaxyl and fosetyl-Al was reported among field isolates of *P. infestans* and *P. cubensis* (COHEN & SAMOUCHA, 1984). These results were neither confirmed in field isolates of *Plasmopara viticola, P. infestans* and *P. cactorum* (BOMPEIX & al., 1984; CLERJEAU & al., 1984) nor in induced mutants of *Phytophthora capsici* (BOWER & COFFEY, 1985).

In this study, cross-resistance to fosetyl-Al was investigated among strains of *Phytophthora capsici* resistant and sensitive to metalaxyl. *P. capsici* mutants resistant to metalaxyl were first induced. Their pathogenicity and sensitivity *in vitro* and *in vivo* to fosetyl-Al were then examined.

MATERIALS AND METHODS

Fungus

Phytophthora capsici Leonian wild-type strain (isolate PC375) was isolated from a naturally-infected pepper (*Capsicum annuum* L.) in Brazzaville (Congo). The pathogen was cultivated on different agar media comprising (per litre) : Cristomalt medium : Cristomalt (DIFAL, France), 10 g, Difco Bacto Agar, 18g; Ribeiro's synthetic medium (RIBEIRO & al., 1975) containing 0.5 mM KH_2PO_4, no β-sitosterol and Difco Bacto Agar; Difco corn meal agar (CMA), 17g; V8 agar medium : 180 ml V8 juice, 3g $CaCo_3$ and 16g agar.

Chemicals

The fungicides utilized were metalaxyl supplied as Ridomil 12% wettable powder, Ciba-Geigy Ltd. (Basel, Switzerland) and fosetyl-Al supplied as Aliette 80% wettable powder, Rhône-Poulenc (Lyon, France).

Induction of mutants resistant to metalaxyl.

A 2 days-old thallus, grown on a Christomalt medium covered with a cellophane membrane (SVIP, France) was exposed to UV light at 254 nm (750, 1000, 1250, 1500 Jm^{-2}). The membrane was transferred either immediately or 48h later to Ribeiro's medium amended with 10 μg/ml metalaxyl. These operations were carried out in darkness to avoid photoreactivation. Petri dishes were then placed in darkness for 15-20 days at a temperature of 22°C.

Pathogenicity of resistant mutants.

The pathogenicity of the wild-type strain and 10 mutants was investigated by inoculating them onto detached tomato leaflets (cv. Roma) from 45 days-old plants. A superficial wound was made on the central vein in the middle of the leaflet. A plug (6 mm in diameter) was taken with a cork-borer from the margin of a 5 days-old culture grown on V8-agar medium and deposited on the wound. After inoculation, the leaflets were transferred to Petri dishes (8,5 cm in diame-

ter) and floated on 10 ml of a MES [2(N-Morpholino)ethanesulfonic acid, Sigma] buffer (22mM, pH 6,5) and incubated at 22°C in continuous light for 72h. The pathogenicities of the wild-type and the mutant strains were evaluated by measuring both the infected areas (using millimetric paper) and the degree of sporulation.

Metalaxyl-resistant isolates were tested on tomato leaflets for tolerance to metalaxyl and fosetyl-Al by amending 20 µg/ml metalaxyl or 200 µg/ml fosetyl-Al in a MES buffer. Pathogenicity was evaluated as described before.

RESULTS

Induction of resistance to metalaxyl.

From day 8 to day 15 after the UV treatment, fast growth rate sectors (FGRS) were initiated from the margin of the culture. The frequency of FGRS was calculated for each level of UV irradiation by the ratio :

$$\frac{\text{number of sectors from treated colonies}}{\text{total number of treated colonies}}$$

This frequency was respectively 40% at 750 Jm^{-2} and 13% at 1000, 1250 and 1500 Jm^{-2}. No FGRS was initiated in the colonies which were not exposed to UV. All sectors tested retained their resistance to metalaxyl after 8-10 days of growth in its absence. Resistance to metalaxyl was evaluated among 10 mutants by measuring the inhibition of radial growth in presence of 10 and 100 µg/ml metalaxyl. The wild-type strain was completely inhibited during 8 days in presence of 10 µg/ml metalaxyl and began to grow very slowly after this period (Fig. 1a). The concentration of 100 µg/ml was lethal for this strain. All mutants except one (SC 150A) grew on 100 µg/ml of metalaxyl.

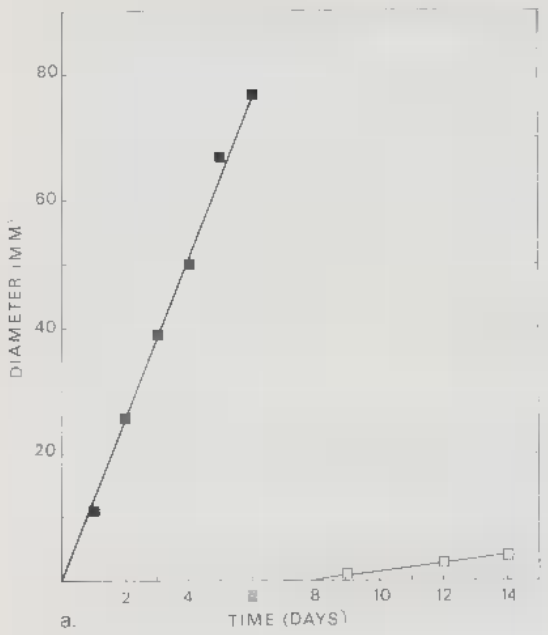
Mutant SC75D grew equally well at 0, 10 and 100 µg/ml metalaxyl (Fig. 1 b) while mutant SC75F growth was inhibited by 50% at both 10 and 100 µg/ml metalaxyl (Fig. 1c).

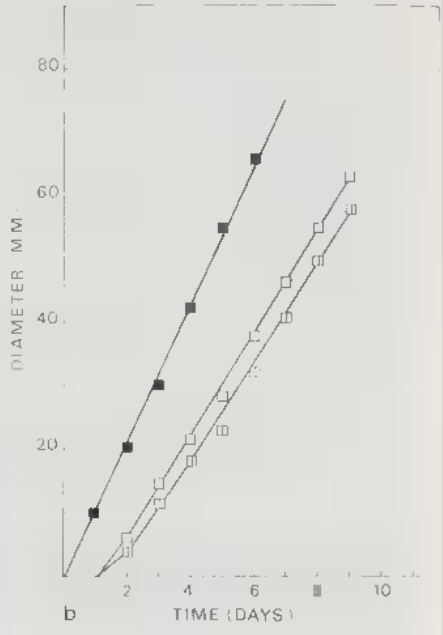
Stability of resistant mutants

After 12 transfers on 10 µg/ml metalaxyl-amended agar, all surviving mutants kept their degree of resistance. The same mutant grown 4 months on agar without metalaxyl were divided into either resistant strains or into strains that lost their resistance partially or entirely.

Pathogenicity and *in vivo* sensitivity of metalaxyl mutants to metalaxyl and fosetyl-Al.

When the mutants were inoculated on tomato leaflets, two types of response were observed according to the foliar surface infected. The first group of mutants (SC75 A, SC75 D, SC75 E, SC100 B) showed pathogenicity similar to that of the wild-type strain in the absence of metalaxyl; lesion size was approxima-





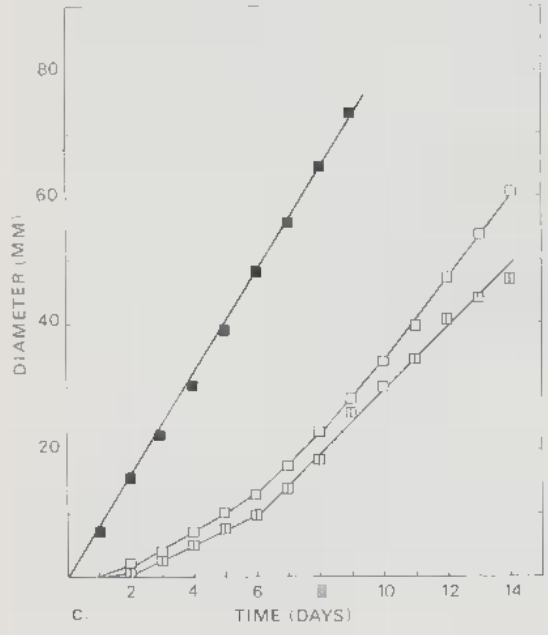


Fig. 1 — Radial growth of *P. capsici* on cristomalt agar (■) supplemented with metalaxyl at 10 µg/ml (□) and 100 µg/ml (▯).
a - wild-type strain, b - mutant SC75 F, c - mutant SC75F.

Fig. 1 — Croissance radiale de *P. capsici* sur cristomalt agar (■) en présence de métalaxyl à 10 µg/ml (□) et 100 µg/ml (▯).
a - souche sauvage, b - mutant SC75 F, c - mutant SC75F.

tely 80-90% of that of the wild-type (Table 1) and sporulation was equivalent. The second group consisted of mutants with pathogenicities that were markedly less reduced and the lesion size was less than 25% of that of the wild-type strain.

Only those mutants with pathogenicities similar to that of the wild-type strain were used in this study.

Table 1. — Effect of metalaxyl and fosetyl-Al on the *in vivo* growth of mutant and wild-type strains of *Phytophthora capsici*.

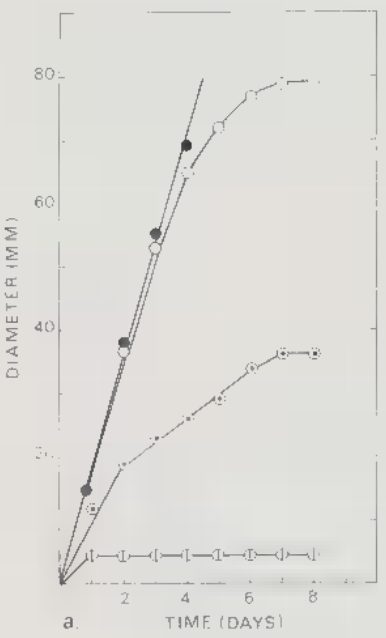
Tableau 1. — Effet du métalaxyl et du phoséthyl-Al sur la croissance *in vivo* des mutants et de la souche sauvage de *Phytophthora capsici*.

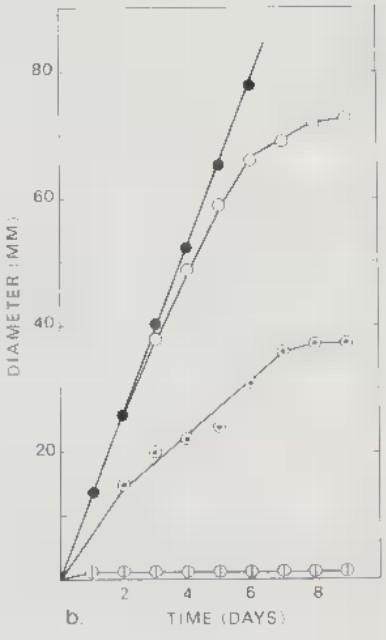
Strains	Foliar surface infected (%)		
	Control[a] (MES, pH 6.5)	Metalaxyl[b] (20 µg/ml)	Fosetyl-Al[b] (200µg/ml)
wild-type	100	0[c]	12,8
SC75A	90,2	3,1	5,5
SC75D	89,1	3,2	7,1
SC75E	90,2	2,2	6,4
SC100B	79,9	1,6	9,4

Detached tomato leaflet were inoculated with 6 mm plugs of *P. capsici*. Incubation on different liquid media was carried out at 22°C in continuous light. Foliar surface infected are measured 72 h after inoculation. Each percentage is calculated with the mean of 15 values. ([a] each value is expressed versus the wild-type strain, [b] each value is expressed versus the corresponding control, [c] necrosis limited to the plug).

Les folioles de tomate en survie sont inoculés avec des implants de 6 mm de *P. capsici*. L'incubation sur différents milieux liquides est réalisée à 22°C en lumière continue. Les surfaces foliaires infectées sont mesurées 72 h après l'inoculation. Chaque pourcentage est calculé à partir de la moyenne de 15 valeurs. ([a] chaque valeur est exprimée par rapport à la souche sauvage, [b] chaque valeur est exprimée par rapport au témoin correspondant, [c] la nécrose est limitée à l'implant).

Detached tomato leaflets were inoculated as previously described and floated for 72h on MES buffer with either metalaxyl (20µg/ml) or fosetyl-Al (200µg/ml) at 22°C in continuous light. The wild-type was completely inhibited in presence of 20 µg/ml metalaxyl (Table 1). By comparison, the four mutants infected a surface area of 12 (1,6%) to 30 mm^2 (3,2%) limited by a distinct border. Wild-type strain and the mutants exhibited a similar behaviour in presence of fosetyl-Al. After an initial progression in the host tissues, the fungal growth was arrested. Infected surface area was nearly 130 mm^2 (12,8%) in the case of the wild-type and 50 to 75 mm^2 (5-9,4%) in the case of mutants. In both cases the infected area was limited by a boundary border.





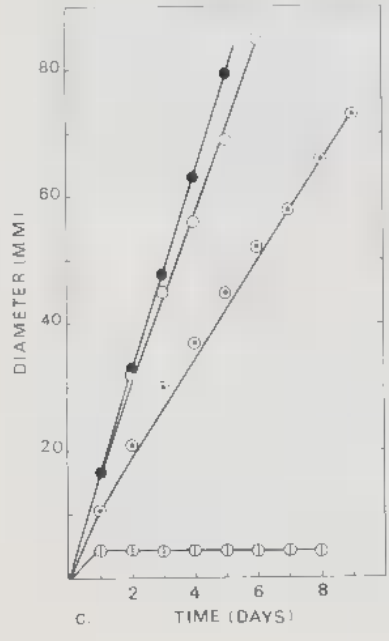


Fig. 2 – Radial growth of *P. capsici* on corn meal agar (CMA) (●) supplemented with fosetyl-Al at 400 µg/ml (○), 800 µg/ml (◉) and 1600 µg/ml (◐).

a - wild-type strain, b - mutant SC75F, c - mutant SC75D.

Fig. 2 – Croissance radiale de *P. capsici* sur «corn meal agar» (CMA) (●) en présence de phosétyl-Al à 400 µg/ml (○), 800 µg/ml (◉) et 1600 µg/ml (◐).

a - souche sauvage, b - mutant SC75F, c - mutant SC75D.

***In vitro* cross-resistance between metalaxyl and fosetyl-Al.**

Inhibition of radial growth of metalaxyl-resistant mutants was recorded on CMA medium containing different fosetyl-Al concentrations (Fig. 2). Among 10 mutants tested, 9 showed a similar response to that of the wild-type. Linear growth on 400 µg/ml fosetyl-Al occurred for 5-6 days, and was followed by a decrease of the growth rate. At the end of the experiment (7-8 days), growth was completely inhibited 5 mm from the edge of the medium. The same phenomenon occurred at 800 µg/ml approximately 7 days after growth initiation.

Only mutant SC75D appeared different from the others in that it grew well on 800 µg/ml; its growth rate was 2 fold greater than the wild-type and no reduction of the growth rate was observed as the colony approached the margin of the agar medium (Fig. 2c).

DISCUSSION

Mutants of *Phytophthora capsici* resistant to metalaxyl were readily obtained by treating thalli with UV radiation. Frequency of mutants recovery was high (nearly 20%). Resistance level of most mutants also was more than 100 µg/ml. Such mutants were also obtained by BRUIN & EDGINGTON (1982) using zoospore mutagenesis. Thallus irradiation on cellophane membrane appears to be an interesting method which can also be used with non or poor spore producers strains of fungi. Moreover, cellophane transfer avoid possible interaction between UV radiation and compounds in the culture medium. This method was only rarely used until now (SENG & al., 1985).

In this study, mutants whose resistance level *in vitro* was more than 100µg/ml metalaxyl were nevertheless inhibited *in vivo* at 20 µg/ml after growth was initiated in host tissue. Two hypotheses can be advanced to explain this phenomenon :

– metalaxyl concentration *in vivo* could locally exceed the DE90 *in vitro*. LAZAROVITZ & WARD (1982) have shown this in the compatible interaction *Phytophthora megasperma* f. sp.*glycinea*/ soybean.

– metalaxyl could partially inhibit mutants growth *in vivo* so that the plant has enough time to defend itself by stimulation of its defense mechanisms. For example, metalaxyl damage could lead the fungus to release cell wall elicitors which could induce a defense reaction (WARD & al., 1980).

This study also shows the absence of cross resistance *in vitro* and *in vivo* between metalaxyl and fosetyl-Al. These results agree with those obtained by other authors (BOMPEIX & al., 1984; CLERJEAU & al., 1984; BOWER & COFFEY, 1985). It has been shown that metalaxyl strongly inhibits RNA-polymerase synthesis (FISHER & HAYES, 1979; DAVIDSE & al., 1981a-b), LAZAROVITZ & WARD, 1982; DAVIDSE & al., 1983). Direct antifungal activity against Oomycetes was demonstrated by several authors (DAVIDSE & al., 1981a-b; SCHWINN, 1983; ZENTMYER, 1983). On the other hand, the mode of action

of fosetyl-Al is still not clearly understood. Indirect action via host metabolism has already been shown (VO-THI-HAI & al., 1979; BOMPEIX & al., 1980, 1981; SAINDRENAN & BOMPEIX, 1986). However, it seems that a direct antifungal action may exist, at least for a number of *Phytophthora* / plant interactions (BOMPEIX & SAINDRENAN, 1984; FENN & COFFEY, 1984; DARAKIS & al., 1985). Until now it is unclear whether resistant strains may exist in the field. At the same time, many strains resistant to anilides were observed *in vivo* and also readily induced *in vitro*. These results tend to show that direct antifungal mode of action of metalaxyl is much more important than for fosetyl-Al Because the chemical structure of the two fungicides is very different, cross-resistance between the two molecules seems improbable except in the case of a pleiotropic mutation affecting different cellular functions. Finally, our results agree with those of BOMPEIX & al. (1984) and CLERJEAU & al. (1984) and show that fosetyl-Al is an effective fungicide against resistant strains to metalaxyl.

ACKNOWLEDGEMENTS

The authors are very grateful to Hedvig ANDERSEN and Rose SCHAEFER for preparation of the manuscript.

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